AssayMax™

Human AT III ELISA Kit

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For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Thank you for choosing Assaypro.

Assay Summary

Step 1. Add 25 µl of Standard or Sample and 25 µl of Biotinylated Protein per well.
Incubate 2 hours.

Step 2. Wash, then add 50 µl of SP Conjugate per well.
Incubate 30 minutes.

Step 3. Wash, then add 50 µl of Chromogen Substrate per well.
Incubate 10 minutes.

Step 4. Add 50 µl of Stop Solution per well.
Read at 450 nm immediately.

Symbol Key

Consult instructions for use.

Assay Template

	A	B	C	D	E	F	G	H
1								
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Human Antithrombin III ELISA Kit

Catalog No. EA3303-1

Sample insert for reference use only

Introduction

The serine protease inhibitor antithrombin III (AT III), the most important natural inhibitor of thrombin activity, has been shown to exert marked anti-inflammatory properties (1). AT III levels are positively correlated with plasma total cholesterol levels, plasma low-density lipoprotein cholesterol levels, plasma triglycerides and D-dimer levels (2).

Principle of the Assay

The AssayMax Human Antithrombin III ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of human AT III in **plasma and serum samples**. This assay employs a quantitative **competitive enzyme immunoassay** technique that measures AT III in less than 3 hours. A polyclonal antibody specific for AT III has been pre-coated onto a 96-well microplate with removable strips. AT III in standards and samples is competed with a biotinylated AT III sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- This product is for **Research Use Only** and is Not For Use In Diagnostic Procedures.
- Prepare all reagents (working diluent buffer, wash buffer, standard, biotinylated protein, and SP conjugate) as instructed, prior to running the assay.
- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- Spin down the SP conjugate vial before opening and using contents.
- The Stop Solution is an acidic solution.
- The kit should not be used beyond the expiration date.

Reagents

- **Human AT III Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against AT III.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes, which can be cut to fit the format of the individual assay.
- **Human AT III Standard:** AT III in a buffered protein base (9.6 µg, lyophilized).
- **Biotinylated Human AT III:** 1 vial, lyophilized.
- **EIA Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate, 100x)**: A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store SP Conjugate at -20°C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.
- Diluent (1x) may be stored for up to 30 days at 2-8°C.
- Store Standard and Biotinylated Protein at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel).
- Deionized or distilled reagent grade water.

Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x *g* for 10 minutes.

Dilute plasma 1:300 with EIA Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).

- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes, and remove serum. Dilute serum 1:300 with EIA Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Refer to Sample Dilution Guidelines below for further instruction.

Guidelines for Dilutions of 1:100 or Greater (*for reference only; please follow the insert for specific dilution suggested)*	
1:100	**1:10000**
A) 4 ul sample: 396 µl buffer(100x) = 100 fold dilution *Assuming the needed volume is less than or equal to 400 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000 fold dilution *Assuming the needed volume is less than or equal to 400 µl.*
1:1000	**1:100000**
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000 fold dilution *Assuming the needed volume is less than or equal to 240 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000 fold dilution *Assuming the needed volume is less than or equal to 240 µl.*

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the EIA Diluent Concentrate 1:10 with reagent grade water. Store for up to 30 days at 2-8°C.
- **Human AT III Standard:** Reconstitute the 9.6 µg of Human AT III Standard with 2.4 ml of EIA Diluent to generate a 4 µg/ml standard stock solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard stock solution (4 µg/ml) 1:2 with EIA Diluent to produce 2, 1, 0.5, 0.25, 0.125, and 0.0625 µg/ml solutions. EIA Diluent serves as the zero standard (0 µg/ml). Any remaining solution should be frozen at -20°C and use within 30 days.

Standard Point	Dilution	[AT III] (µg/ml)
P1	1 part Standard (4 µg/ml)	4.0000
P2	1 part P1 + 1 part EIA Diluent	2.0000
P3	1 part P2 + 1 part EIA Diluent	1.0000
P4	1 part P3 + 1 part EIA Diluent	0.5000
P5	1 part P4 + 1 part EIA Diluent	0.2500
P6	1 part P5 + 1 part EIA Diluent	0.1250
P7	1 part P6 + 1 part EIA Diluent	0.0625
P8	EIA Diluent	0.0000

- **Biotinylated Human AT III (6x):** Reconstitute Biotinylated Human AT III with 4 ml EIA Diluent to produce a 6-fold stock solution. Allow the biotin to sit for 10 minutes with gentle agitation prior to making dilutions. The stock solution should be further diluted 1:6 with EIA Diluent. Any remaining solution should be frozen at -20°C and used within 30 days.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with EIA Diluent. Any remaining solution should be frozen at -20°C.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 25 µl of Human AT III Standard and/or sample per well and immediately add 25 µl of Biotinylated Human AT III to each well (on top of the standard or sample) and tap plate gently to mix. Cover wells with a sealing tape and incubate for 2 hours at room temperature. Start the timer after the last addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 µl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 µl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

- Wash the microplate as described above.
- Add 50 μl of Chromogen Substrate per well and incubate for 10 minutes or until the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 μl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at low concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard, and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using 4-parameter or log-log logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

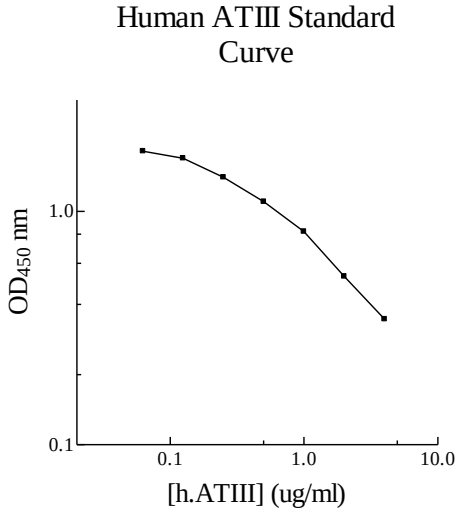
Typical Data

- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	µg/ml	OD	Average OD
P1	4.0000	0.343 0.346	0.345
P2	2.0000	0.544 0.510	0.527
P3	1.0000	0.820 0.819	0.820
P4	0.5000	1.257 1.166	1.211
P5	0.2500	1.403 1.399	1.401
P6	0.1250	1.691 1.687	1.689
P7	0.0625	1.811 1.814	1.812
P8	0.0000	2.181 2.179	2.180
Sample: Human Pool Normal, Sodium Citrate Plasma (300x)		0.818 0.839	0.828

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Reference Value

- Human plasma and serum samples from healthy adults were tested (n=40). On average, AT III level was 290 µg/ml.

Sample	n	Average Value (µg/ml)
Human Pool Normal Plasma	10	297
Human Normal Plasma	20	269
Human Pool Normal Serum	10	305

Performance Characteristics

- The minimum detectable dose of AT III as calculated by 2SD from the mean of a zero standard was established to be 0.03 µg/ml.
- Intra-assay precision was determined by testing replicates of three plasma samples in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	3.1%	3.2%	3.6%	8.9%	9.0%	9.1%
Average CV (%)	3.3%			9.0%		

Recovery

Standard Added Value	0.1 – 1 µg/ml
Recovery %	89 – 108%
Average Recovery %	96.5%

Linearity

- Plasma and serum samples were serially-diluted to test for linearity.

Average Percentage of Expected Value (%)		
Sample Dilution	Plasma	Serum
1:150	106%	105%
1:300	97%	98%
1:600	95%	96%

Cross-Reactivity

Species	Cross Reactivity (%)
Canine	None
Monkey	<10%
Mouse	None
Rat	None
Swine	None
Rabbit	None
Bovine	None
Human	100%

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of expired components	• Check the expiration date listed before use. • Do not interchange components from different lots.
	Improper wash step	• Check that the correct wash buffer is being used. • Check that all wells are dry after aspiration. • Check that the microplate washer is dispensing properly. • If washing by pipette, check for proper pipetting technique.
	Splashing of reagents while loading wells	• Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.
	Improperly sealed microplate	• Check the microplate pouch for proper sealing. • Check that the microplate pouch has no punctures. • Check that three desiccants are inside the microplate pouch prior to sealing.
Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	• Each step of the procedure should be performed uninterrupted.
	Omission of step	• Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	• Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	• Check pipette calibration. • Check pipette for proper performance.
	Wash step was skipped	• Consult the provided procedure for all wash steps.
	Improper wash buffer	• Check that the correct wash buffer is being used.
	Improper reagent preparation	• Consult reagent preparation section for the correct dilutions of all reagents.
	Insufficient or prolonged incubation periods	• Consult the provided procedure for correct incubation time.

Deficient Standard Curve Fit	Non-optimal sample dilution	• Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay. • Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay. • User should determine the optimal dilution factor for samples.
	Contamination of reagents	• A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	• Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	Improper pipetting	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.

References

(1) Oelschläger C *et al.* (2002) *Blood* 99(11):4015-20.
(2) Erem C *et al.* (2005) *Med Princ Pract.* 14(1): 22-30

Version 3.1R

Related Products

- EA3301-1 AssayMax Human AT III ELISA Kit (Urine, Saliva, Milk, and Cell Culture samples)
- EMA3301-1 AssayMax Mouse AT III ELISA Kit (Plasma, Serum, Urine, and Cell Culture samples)